(12) UK Patent Application (19) GB (11) 2 092 596 A

(21) Application No 8138588
(22) Date of filing 22 Dec 1981
(30) Priority data
(31) 55/187011
(32) 30 Dec 1980
(33) Japan (JP)
(43) Application published
18 Aug 1982
(51) INT CL³
C07G 15/00
(52) Domestic classification
C3H FX
(56) Documents cited
GBA 2040292
GBA 2016015
(58) Field of search
C3H
(71) Applicants
Kabushiki Kaisha
Hayashibara Seibutsu
Kagaku Kenkyujo,
2—3 1-chome,
Shimoishii, Okayama-shi,
Okayama, Japan
(72) Inventor
Kaname Sugimoto
(74) Agents
Page, White and Farrer,
27, Chancery Lane,
London WC2A 1NT

(54) **Process for the production of human parathyroid hormone**

(57) A process for the production of human parathyroid hormone (hPTH), comprising *in vivo* multiplication of human lymphoblastoid cells capable of producing said hormone, using a non-human warm-blooded animal, and *in vitro* cultivation of the multiplied human lymphoblastoid cells to produce hPTH.

GB 2 092 596 A

SPECIFICATION

Process for the production of human parathyroid hormone

The present invention relates to a process for the production of human parathyroid hormone (abbreviated as hPTH hereinafter).

hPTH is a hormone, secreted by the parathyroid glands, that regulates the synthesis of activated vitamin D_3, stimulates the release of calcium from bones and leads to an increase in the calcium level of the blood. No process for the mass production of low-cost hPTH has been established to date.

We have investigated processes for the mass production of hPTH and have unexpectedly found that certain human lymphoblastoid cells capable of producing hPTH are suitable for the mass production of hPTH owing to their very high multiplication efficiency and high rate of hPTH production per cell.

According to the present invention there is provided a process for the production of hPTH, which process comprises multiplying human lymphoblastoid cells capable of producing said hormone by transplanting said cells to a non-human warm-blooded animal body, or alternatively by allowing said cells to multiply within a device in which the nutrient body fluid of a non-human warm-blooded animal is supplied to said cells, and allowing the human lymphoblastoid cells multiplied by either of the above multiplication procedures to release said hormone.

The process according to the invention provides an extremely high hPTH yield, requires much less nutrient medium containing expensive serum for the cell multiplication or no such medium, and renders much easier the maintenance of the culture medium during the cell multiplication than in *in vitro* tissue culture. Particularly, any human lymphoblastoid cells capable of producing hPTH can be multiplied easily while utilising the nutrient body fluid supplied from the non-human warm-blooded animal body by transplanting said cells to the animal body, or suspending the cells in a conventional diffusion chamber devised to receive the nutrient body fluid of the animal, and feeding the animal in the usual way. Also, in the present process one obtains a more stable and a higher rate of cell multiplication, and a higher hPTH production per cell.

As regards the human lymphoblastoid cells which may be used in the present invention, any human lymphoblastoid cells can be used as long as they produce hPTH and multiply rapidly in the non-human warm-blooded animal body. Preferable human lymphoblastoid cells are those introduced with hPTH production governing genetic sites of human cells which inherently produce hPTH such as normal or transformed parathyroid cells, or human cells which produce ectopic hPTH such as lung carcinoma cells, ovarian tumor cells, kidney carcinoma cells or liver carcinoma cells by means of cell fusion using polyethylene glycol, or by genetic recombination techniques using DNA ligase, nuclease and DNA polymerase; and other human lymphoblastoid cells which produce ectopic hPTH. Since the use of such human lymphoblastoid cells results in the formation of easily disaggregatable massive tumors when the cells are transplanted to the animal body, and the massive tumors are barely contaminated with the host animal cells, the multiplied live human lymphoblastoid cells can be harvested easily.

Any non-human warm-blooded animal can be used to perform the process of the present invention as long as the human lymphoblastoid cells multiply therein. Examples of suitable animals are poultry such as chickens or pigeons, and mammals such as dogs, cats, monkeys, goats, pigs, cows, horses, guinea pigs, rats, hamsters, mice and nude mice. Since transplantation of the human lymphoblastoid cells gives rise to undesirable immunoreactions, the use of a newborn or infant animal, or of an animal in the youngest possible stage, for example, in the form of an egg, embryo or foetus, is desirable. In order to reduce the incidence of immunoreactions as much as possible, prior to the cell transplantation the animal may be treated with X-ray of γ-ray irradiation, at about 200—600 rem, or with an injection of antiserum or an immunosuppressive agent prepared according to conventional methods. Nude mice exhibit weak immunoreactions; consequently, any human lymphoblastoid lines capable of producing hPTH can advantageously be transplanted into, and rapidly multiplied in, nude mice without subjecting the mice to a pretreatment for suppressing immunoreactions.

Stabilised cell multiplication and enhancement of hPth production can be both carried out by repeated transplantation using combination(s) of different non-human warm-blooded animals; the objectives are attainable first by implanting said cells in hamsters and multiplying the cells therein, then by reimplanting the cells in nude mice. Repeated transplantation may be carried out with animals of the same class or division as well as those of the same species or genus.

The human cells to be multiplied can be implanted in any site of the animal as long as they multiply at that site; for example, in the allantoic cavity, or intravenously, intraperitoneally, or subcutaneously.

In addition to the above-mentioned direct cell transplantation, any conventional human lymphoblastoid lines capable of producing hPTH can be multiplied easily by using the nutrient body fluid supplied from the animal body by embedding, for example, intraperitoneally, in the animal body a conventional diffusion chamber, of various shapes and sizes, and equipped with a porous membrane filter, ultra filter or hollow fiber having a pore size of about 10^{-7} to 10^{-5} m in diameter which prevents ingress of the host animal cells into the chamber but permits the cells to be supplied with the nutrient body fluid of the animal. Furthermore, the diffusion chamber can be designed, if desired, so as to enable observation of the cell suspension

in the chamber through transparent side window(s) provided on the chamber wall(s), and so as to enable replacement and exchange with a fresh chamber. In this way cell production per host can be increased to even higher levels over the period of the animal's life without any sacrifice of the host animal. When such a diffusion chamber is used, since little immunoreaction arises owing to the absence of direct contact of the human cells with the host animal cells, the multiplied human lymphoblastoid cells can be harvested easily, and any non-human warm-blooded animal can be used as the host in the present process without the need for any pretreatment to reduce immunoreactions.

Feeding of the host animal can be carried out by conventional methods even after cell transplantation, and no special care is required.

Maximum cell multiplication can be attained within 1—20 weeks, generally 1—5 weeks, after the cell transplantation.

According to the invention, the number of the human lymphoblastoid cells obtained per host ranges from about 10^7 to 10^{12} or more. In other words, the number of the human lymphoblastoid cells implanted in the animal body increases about 10^2 to 10^7 times or more, or about 10^1 to 10^6 times or more than that attained by *in vitro* tissue culture method using a nutrient medium; the human lymphoblastoid cells can therefore be used advantageously in the production of hPTH.

As regards the method by which the human lymphoblastoid cells multiplied by either of the above described procedures are allowed to release hPTH, any methods can be employed as long as the said human cells release the desired hormone thereby. For example, human lymphoblastoid cells, obtained by multiplying in ascite in suspension and harvesting from said ascite, or by extracting the massive tumor formed subcutaneously and harvesting after the disaggregation of the massive tumor, are suspended to give a cell concentration of about 10^4 to 10^8 cells per ml in a nutrient medium, prewarmed at a temperature of about 20—40°C, and then incubated at this temperature for about 1 to 100 hours to produce hPTH. During the incubation, enhancement of hPTH production may be carried out by including one or more of an amino acid such as glycine, leucine, lysine, arginine and cysteine; an inorganic salt such as sodium chloride, potassium chloride, calcium chloride and magnesium sulfate; and a hormone such as dopamine, isoproterenol, epinephrine and norepinephrine.

The hPTH thus obtained can be collected easily by purification and separation techniques using conventional procedures such as salting-out, dialysis, filtration, centrifugation, concentration and lyophilisation. If a more highly purified hPTH preparation is desirable, a preparation of the highest purity can be obtained by the above-mentioned techniques in combination with other conventional procedures such as adsorption and desorption with ion exchange, gel filtration, affinity chromatography, isoelectric point fractionation and electrophoresis.

The hPTH preparation thus obtained can be used advantageously alone or in combination with one or more agents for injection, or for external, internal or diagnostical administration in the prevention and treatment of human diseases.

The following Examples illustrate the present invention.

In this specification, hPTH production was determined by a bioassay method as described in J. A. Parsons *et al.*, Endocrinology, Vol 92, pp. 454—462 (1973), and is expressed by weight in terms of the standard hPTH preparation, assigned 1,300 USP units per mg, available from the National Institute of Health (USA).

EXAMPLE 1

Disaggregated human parathyroid tumor cells — extracted from a patient suffering from parathyroid tumor and minced — and a human leukemic lymphoblastoid line Namalwa were suspended together in a vessel with a salt solution, containing 140 mM NaCl, 54 mM KCl, 1 mM NaH_2PO_4 and 2 mM $CaCl_2$, to give a respective cell concentration of about 10^6 cells per ml. The ice-chilled cell suspension was mixed with a fresh preparation of the same salt solution containing UV-irradiation preinactivated Sendai virus, transferred into a 37°C incubator five minutes after the mixing, and stirred therein for 30 minutes to effect cell fusion, thereby introducing the ability of the human parathyroid tumor cells of producing hPTH into the human leukemic lymphoblastoid line.

After cloning according to conventional methods the hybridoma cell strain capable of producing hPTH, the hybridoma cells strain was implanted intraperitoneally in adult nude mice which were then fed in the usual way for five weeks. The resulting massive tumors, about 15 g each, were extracted and disaggregated by mincing and trypsinizing.

After washing the cells with Earle's 199 medium (pH 7.2), supplemented with 10 v/v % foetal calf serum, the cells were resuspended to give a cell concentration of about 10^5 cells per ml in a fresh preparation of the same medium which contained 30 mM L-arginine and 20 mM $CaCl_2$, and then incubated at 37°C for 40 hours to produce hPTH. Thereafter, the cells were treated ultrasonically, and the hPTH in the resulting supernatant was determined. The hPTH production was about 830 ng per ml cell suspension.

Control cells were obtained by cultivating *in vitro* the human parathyroid cells at 37°C in Earle's 199 medium (pH 7.2), supplemented with 10 v/v % foetal calf serum. These cells were treated similarly as described above to produce hPTH. The hPTH production was only about 4 ng per ml cell suspension.

EXAMPLE 2

Disaggregated human kidney carcinoma cells — extracted from a patient suffering from

kidney carcinoma and minced — and a human leukemic lymphoblastoid line JBL were fused in a manner similar to that described in Example 1, thereby introducing the ability of the human kidney carcinoma cells of producing hPTH into the human leukemic lymphoblastoid line.

After cloning according to conventional methods the hybridoma cell strain capable of producing hPTH, the hybridoma cell strain was implanted subcutaneously in newborn hamsters which had been preinjected with an antiserum (prepared from rabbits using conventional methods) so as to reduce the immunoreactions of the rabbits. The rabbits were then fed in the usual way for three weeks.

The resulting massive tumors, formed subcutaneously and about 10 g each, were extracted and disaggregated by mincing and suspending in a physiological saline solution containing collagenase.

After washing the cells with Eagle's minimal essential medium (pH 7.4), supplemented with 5 v/v % human serum, the cells were resuspended to give a cell concentration of about 10^6 cells per ml in a fresh preparation of the same medium which contained 20 mM $CaCl_2$ and 20 mM dopamine, and then incubated at 37°C for 20 hours to produce hPTH. The hPTH production was about 1.3 μg per ml cell suspension.

Control cells were obtained similarly as described in Example 1 by cultivating *in vitro* the fused human leukemic lymphoblastoid line JLB. The control cells were treated similarly as described above. The hPTH production was only about 16 ng per ml cell suspension.

EXAMPLE 3

Newborn rats were implanted intravenously with a human leukemic lymphoblastoid line BALL-1 into which the ability of human ovarian tumor cells of producing hPTH had been introduced in a manner similar to that described in Example 1, and then fed in the usual way for four weeks.

The resulting massive tumors, about 30 g each, were extracted and treated similarly as described in Example 1 to produce hPTH. The hPTH production was about 900 ng per ml suspension.

Control cells were obtained similarly as described in Example 1 by cultivating *in vitro* the fused human leukemic lymphoblastoid line BALL-1. These control cells were treated similarly as described above. The hPTH production was only about 10 ng per ml cell suspension.

EXAMPLE 4

After about 400 rem X-ray irradiation of adult mice to reduce their immunoreactions, the mice were implanted subcutaneously with a human leukemic lymphoblastoid line NALL-1 into which the ability of human lung carcinoma cells to produce hPTH had been introduced in a manner similar to that described in Example 1. The mice were then fed in the usual way for three weeks.

The resulting massive tumors, formed subcutaneously and about 15 g each, were extracted and treated similarly as described in Example 2 to produce hPTH. The hPTH production was about 1.2 μg per ml cell suspension.

Control cells were obtained similarly as described in Example 1 by cultivating *in vitro* the fused human leukemic lymphoblastoid line NALL-1. The control cells were treated similarly as described above. The hPTH production was only about 20 ng per ml cell suspension.

EXAMPLE 5

A human leukemic lymphoblastoid line TALL-1 into which the ability of the human parathyroid tumor cells to produce hPTH had been introduced in a manner similar to that described in Example 1 was suspended in physiological saline solution, and the resulting cell suspension was transferred into a plastic cylindrical diffusion chamber (inner volume: about 10 ml) and equipped with a membrane filter having a pore size of about 0.5 μ in diameter. After intraperitoneal embedding of the chamber into an adult rat, the rat was fed in the usual way for four weeks, and the chamber was removed.

The human lymphoblastoid cell density in the chamber attained by the above operation was about 6×10^8 cells per ml which was about 10^2 times higher or more than that attained by *in vitro* cultivation using a CO_2 incubator. The human lymphoblastoid cells thus obtained were treated similarly as described in Example 2 to produce hPTH. The hPTH production was about 1.1 μg per ml cell suspension.

Control cells were obtained by suspending the human parathyroid tumor cells in physiological saline solution, transferring the resulting cell suspension in the chamber, embedding intraperitoneally the chamber into an adult rat, feeding the rat in the usual way for four weeks, and harvesting the multiplied human lymphoblastoid cells (human cell density, about 8×10^6 cells per ml). The control cells were treated similarly as described above. The hPTH production was only about 3 μg per ml cell suspension.

EXAMPLE 6

A human leukemic lymphoblastoid line JBL into which the ability of the human lung carcinoma cells to produce hPTH had been introduced in a manner similar to that described in Example 1 was implanted in the allantoic cavities of embryonated eggs which had been preincubated at 37°C for five days. After further incubation of the eggs at this temperature for an additional one week, the chamber was removed.

The multiplied human lymphoblastoid cells thus obtained were treated similarly as described in Example 1 to produce hPTH. The hPTH production was about 700 ng per ml cell suspension.

In a control experiment in which the human lung carcinoma cells were implanted in the allantoic cavities of embryonated eggs, no cell multiplication was observed.

CLAIMS

1. A process for the production of human parathyroid hormone (hPTH), which process comprises multiplying human lymphoblastoid cells capable of producing said hormone by transplanting said cells to a non-human warm-blooded animal body, and allowing the multiplied human lymphoblastoid cells to release said hormone; or multiplying human lymphoblastoid cells capable of producing said hormone by allowing said cells to multiply within a device in which the nutrient body fluid of a non-human warm-blooded animal is supplied to said cells, and allowing the multiplied human lymphoblastoid cells to release said hormone.

2. A process according to Claim 1, wherein the human lymphoblastoid cells are hybridoma cells obtained by cell fusion of a human lymphoblastoid line with human cells capable of producing hPTH.

3. A process according to Claim 2, wherein the hybridoma cells are those obtained by cell fusion of a human lymphoblastoid line with human parathyroid tumor cells.

4. A process according to Claim 2, wherein the hybridoma cells are those obtained by cell fusion of a human lymphoblastoid line with human kidney tumor cells.

5. A process according to Claim 2, wherein the hybridoma cells are those obtained by cell fusion of a human lymphoblastoid line with human ovarian tumor cells.

6. A process according to Claim 2, wherein the hybridoma cells are those obtained by cell fusion of a human lymphoblastoid line with human lung carcinoma cells.

7. A process according to any one of Claims 2 to 6 wherein the human lymphoblastoid line is a human leukemic lymphoblastoid line.

8. A process according to any one of Claims 2 to 7, wherein the human lymphoblastoid line is Namalwa, BALL-1, NALL-1, TALL-1 or JBL.

9. A process according to any one of the preceding Claims, wherein the multiplied human lymphoblastoid cells are allowed to release hPTH in the presence of one or more of glycine, leucine, lysine, arginine, cysteine; sodium chloride, potassium chloride, calcium chloride, magnesium sulfate; dopamine, isoproterenol, epinephrine and norepinephrine.

10. A process according to any one of the preceding claims, wherein the non-human warm-blooded animal is a chicken, pigeon, dog, cat, monkey, goat, pig, cow, horse, guinea pig, rat, hamster, mouse or nude mouse.

11. A process according to Claim 1 substantially as hereinbefore described in any one of the Examples.

12. Human parathyroid hormone whenever prepared by a process as claimed in any one of the preceding claims.

Printed for Her Majesty's Stationery Office by the Courier Press, Leamington Spa, 1982. Published by the Patent Office, 25 Southampton Buildings, London, WC2A 1AY, from which copies may be obtained.